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Theileria annulata* induces aberrant T cell activation *in vitro* and *in vivo

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SUMMARY

The protozoan parasite of cattle, *Theileria annulata*, causes a severe lymphoproliferative disease, developing initially in the draining lymph node, which is often fatal in naive animals. Infection of macrophages with *T. annulata* leads to an augmentation of their antigen-presenting capability *in vitro* and infected cells can induce proliferation of autologous resting T cells from naive animals. This inappropriate activation of T cells may play an important role in the failure of the host to mount an effective immune response *in vivo*. To investigate this hypothesis we characterized further the response of T cells from naive cattle to infected cells *in vitro*, and also examined the development of the immune response in lymph nodes draining the sites of *T. annulata* infection. Both CD4⁺ and CD8⁺ T cells from naive peripheral blood mononuclear cells (PBMC) were induced to proliferate and express the activation markers IL-2R and MHC class II when cultured with infected cells. This effect was seen in both 'naive' and 'memory' T cells, and was dependent upon contact with infected cells. *In vitro*, infected cells are therefore capable of activating T cells irrespective of their antigen specificity or memory status. In draining lymph nodes, although large numbers of IL-2R⁺ cells developed following infection, these activated cells were only associated with areas of parasite-induced proliferating cells, and subsequently disappeared from the node. Cells expressing IL-2R were not present in recognized sites for T cell development. Germinal centres were severely affected, losing T cell-dependent zones followed by a total destruction of morphology. T cell function is therefore severely disrupted within draining nodes. This study has shown that parasitized cells supply sufficient signals *in vitro* to activate T cells irrespective of specificity. T cells also are not stimulated in a conventional manner *in vivo*, and this may play an important role in preventing an effective immune response from being generated.

Keywords *Theileria annulata* T cells antigen presentation activation markers

INTRODUCTION

Theileria annulata is a tick-transmitted protozoan parasite, affecting cattle in Southern Europe, North Africa, India, the Middle East and Southern Russia, causing tropical theileriosis. Little is known about the primary immune response to *T. annulata* in naive animals, where infection quickly leads to death. Parasite development takes place within the lymph node draining the site of initial infection, and is therefore in a position to interfere with immune response development. When infected animals are treated with the drug 'butalex', which kills the intracellular parasite, the animal recovers [1] and is subsequently immune. Animals recovering from infection generate anti-parasite infected cell CD8⁺ cytotoxic T lymphocytes (CTL) [2,3]. This restoration of immune function suggests that although the host is capable of generating an immune response, the parasite infection overwhelms normal immunity.

In vitro it has been previously shown that *T. annulata* preferentially infects cells of monocyte/macrophage lineage [4,5], and transforms them into continuously growing cell lines. These macroschizont infected cells can act as antigen-presenting cells (APC) and present third party peptides via MHC class II to antigen-specific CD4⁺ T cells *in vitro* [6]. Infected cells exhibit augmented APC function, inducing greater proliferation to peptide than normal APC. In addition, infected cells also induce 'non-specific' proliferation of autologous resting T cells from naive cattle in the absence of any exogenously added antigen [6,7]. These observations suggest that infected cells may have an inherent capability to activate T cells. We postulate that inappropriate activation of T cells by parasitized cells may explain the inability of the host to mount an effective immune response. Here we have characterized the activation effects of infected cells upon naive cells *in vitro*. We have also investigated whether this phenomenon affects immunity *in vivo*, by examining the development of the primary immune response to *T. annulata* in draining lymph nodes.

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MATERIALS AND METHODS

Animals

Hereford calves aged approximately 4 months were used for the examination of draining lymph nodes. All other animals used in this study were normal Friesian female or castrated male cattle aged 6 months or older.

Immunizations

Theileria annulata sporozoites were prepared from infected ticks, prepared as previously described [8]. Lymph nodes were obtained from two calves infected with percoll-purified sporozoites of *T. annulata* [9] (Ankara strain). A third control calf was immunized with the appropriate percoll fraction from uninfected *Hyalomma* ticks. In addition, the draining lymph nodes from two calves infected with unpurified Gharb strain sporozoites were also examined 10 days post-infection.

Theileria annulata-infected cell lines

Macroschizont infected cell lines (Hissar or Gharb strain) from the animals tested were prepared as previously described [6]. Cell lines were used at low passage number (2–20).

Cell preparation

Peripheral blood mononuclear cells (PBMC) were separated using Ficoll-Hypaque ('Lymphoprep', Nycomed, Oslo, Norway) as previously described [10]. Complete tissue culture medium was used throughout the experiments [6].

Culture of PBMC

PBMC were cultured with *T. annulata*-infected cells essentially as described previously [6]. Briefly, PBMC were cultured with autologous irradiated (75 Gy) infected cells in 6 × 10 ml well plates (Nunc, Gibco, Paisley, UK). PBMC (8 × 10⁶/ml): infected cell ratios varied from 10:1 to 40:1. In addition, PBMC (2.5 × 10⁶/ml) were stimulated with 5 µg/ml concanavalin A (Con A) to provide a 'control' population of stimulated T cells. Identical cultures with PBMC separated from infected cells or from medium containing Con A by a 0.4-µm pore membrane (Millicel-CM Insert, Millipore Corp., Bedford, MA) were also established in six-well plates. Stimulated PBMC were harvested at various times (1–7 days) for FACS analysis.

Proliferation assays

Proliferation assays were performed as previously described [6]. Briefly, PBMC (4 × 10⁵/well) were incubated with irradiated autologous infected cells (2 × 10⁴/well) or 5 µg/ml Con A for 1–9 days. Proliferation was measured using liquid scintillation following a 6-h pulse of ³H-dThd (Amersham, Aylesbury, UK). As the membrane inserts were too large to use in 96-well plates, cells grown in the presence of inserts in six-well plates for the desired length of time were plated out in 96-well plates at the same dilution as normal assays, pulsed, and counted.

MoAbs

MoAbs used in these experiments are detailed in Table 1. MoAbs were used at optimal concentrations (1:1000 dilution of ascitic fluid or 1:20 dilutions of culture supernatant) for FACS analysis. The use of MoAb for staining sections is detailed under immunohistology.

Table 1.

MoAb	Specificity
IL-A12	Bovine CD4 [11]
J11	Bovine MHC class II [12]
IL-A111	Bovine IL-2 receptor [13]
CC63	Bovine CD8 [14]
1C7	<i>T. annulata</i> macroschizonts [15]
VPM 30	Pan B cells in peripheral blood, germinal centres [16]
CC76	Bovine CD45RB [14]
MIB-1	Ki-67 proliferation antigen [17]

FACS analysis

Cells were analysed using the MoAbs described above on a Becton Dickinson FACScan essentially as previously described [10]. Double staining was performed by first incubating with both MoAbs (of different isotypes) followed by appropriate fluorescent secondary reagents: goat anti-mouse (GAM) IgM-FITC; GAM IgG-PE (Sigma, Poole, UK); GAM IgG1-FITC; GAM IgG2a-PE (Seralab).

Lymph nodes

The right prescapular lymph nodes (draining the site of infection) were taken from the control and one infected animal at day 4 post-infection. Nodes were taken from the other percoll-purified sporozoite-infected animal at day 8, and from the two Gharb-infected animals at day 10. Pieces of node approx. 0.5–1 cm² were fixed in 10% formal buffered saline pH 7.4 and subsequently embedded in paraffin wax. Sections (2.5–3 µm) were cut from paraffin wax-embedded lymph nodes. Sections were stained with haematoxylin and eosin (H&E) or Giemsa for conventional histological examination. Infection with macroschizonts was assessed in Giemsa-stained needle biopsies from the nodes.

Immunohistology

Sections were stained with the MoAbs detailed above. Optimal concentrations of MoAbs were determined and used in the range neat–1:10 dilutions of culture supernate or as 1:50–1:100 dilutions of ascites.

MoAbs staining of formalin-fixed sections was performed using standard immunohistochemical techniques. Sections were dewaxed in xylene and rehydrated through alcohol. MoAbs IL-A111, J11, and IL-A12 required the sections to be treated with trypsin digestion solution for 20 min at 37°C before staining. (Digestion solution: 0.1% w/v Trypsin 1:250 (Difco, Detroit, MI) and 0.1% CaCl₂ (BDH, Poole, UK) in distilled H₂O.) Staining was visualized using the ABC-AP system (Dako, Glostrup, Denmark) with Vector red (Vector Labs, Peterborough, UK) as the substrate. Vector red has the advantage of being visible by both conventional and ultraviolet/confocal microscopy.

RESULTS

Activation of T cells from naive animals

Previous studies have shown that naive T cells proliferated to infected cells, and that the magnitude of proliferation was proportional to the ratio infected cells:PBMC or T cells [6,7].

In order to analyse this response and phenotype the responding cells, PBMC from naive animals were cultured with irradiated autologous infected cells. PBMC were also incubated with Con A as a positive activation control. Increasing the numbers of infected cells:PBMC in these experiments again led to increased proliferation (results not shown). The results presented here used infected cells:PBMC ratios of 1:20, except where indicated.

Both infected cells and Con A induced proliferation, although the time courses were dissimilar (Fig. 1). With infected cells, proliferation peaked after 5 days as described previously [6], whereas with Con A proliferation peaked much earlier at day 2. In addition the amount of proliferation induced by infected cells was always substantially less than with Con A.

Incubation with infected cells increased the numbers of CD4⁺ and CD8⁺ T cells expressing IL-2R and MHC class II molecules on their surface by approximately 25% (Fig. 2). These determinants were also expressed upon the surface of Con A-activated T cells (Fig. 2). IL-2R and MHC class II expression by T cells was detected after 24 h of culture, plateaued by 48 h, and was stable for up to 7 days (Fig. 3). Increasing the ratio of infected cells:PBMC to 1:10 led to increased expression of IL-2R and MHC class II upon CD4⁺ and CD8⁺ cells (48 h timepoint shown in Fig. 3). IL-2R and MHC class II were expressed upon both CC76 (CD45RB) 'high' and 'low' staining cells (results not shown). Separating PBMC from the infected cells by a 0.4- μ m membrane completely blocked the expression of any of the activation associated markers on T cells (Fig. 2), but had no effect upon Con A stimulation (results not shown).

Draining lymph nodes

Theileria annulata macroschizonts usually appear in the draining lymph node 5 days post-infection, reaching a peak

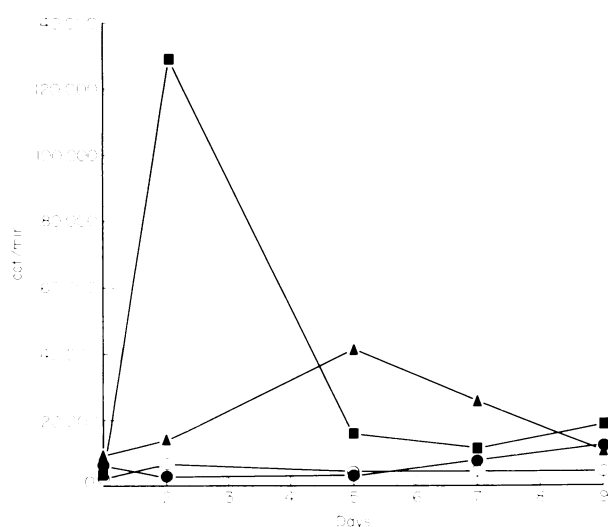


Fig. 1. Proliferative responses of peripheral blood mononuclear cells (PBMC) from a naive animal to infected cells or concanavalin A (Con A). c.c.t./min. Corrected counts per minute. Values are means of quadruplicate samples. ○, PBMC; ●, *Theileria annulata*; ▲, PBMC + *T. annulata*; ■, Con A 5 μ g.

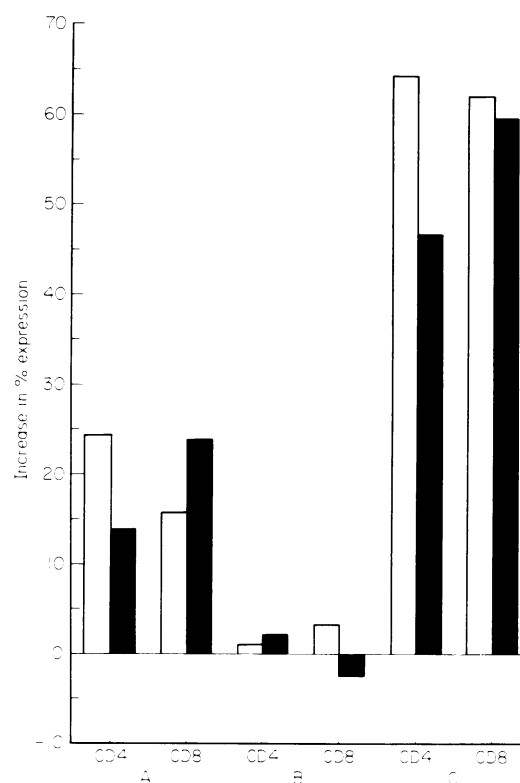


Fig. 2. Changes from control levels of expression of IL-2 receptors (□) and MHC class II (■) by bovine T cells in peripheral blood mononuclear cells (PBMC) after 48 h culture with: A, infected cells (1:20); B, infected cells (1:20) separated from the PBMC by a 0.4- μ m membrane; C, concanavalin A (Con A) 5 μ g/ml. Results are means from four different animals.

at days 8–9, with European cattle rarely surviving beyond day 10 or 11 (C.G.D. Brown, personal communication). Here we examined draining lymph nodes at days 4, 8 and 10 in order to study the immune response before macroschizonts are detectable, and during the period when parasite growth is at its peak. The description of the pathology of *T. annulata* infection within draining lymph nodes is limited to specific points concerned with the induction of the immune response.

Macroschizonts were not present in control or day 4 Giemsa-stained biopsies, and day 8 biopsies contained <1% macroschizonts. Lymph nodes removed from the two animals at day 10 contained 27% and 53% macroschizonts, respectively, within the biopsies, and most cells within sections of these nodes were 1C7⁺.

The percoll control draining node showed no signs of reaction, with normal germinal centre morphology, small lymphocytes within the cortex, and empty medullary sinuses. The most striking feature of the *T. annulata* infection was the appearance of foci of large blasting cells in the medulla of the day 4 draining node, spreading throughout the tissue by day 8 (Fig. 4a). On day 10, these were the predominant cell type present, all normal morphology having disappeared from the nodes. The blasting cells stained strongly with MoAb MIB-1 (anti-proliferation antigen) and anti-MHC class II MoAb. Studies of frozen sections from infected animals have shown

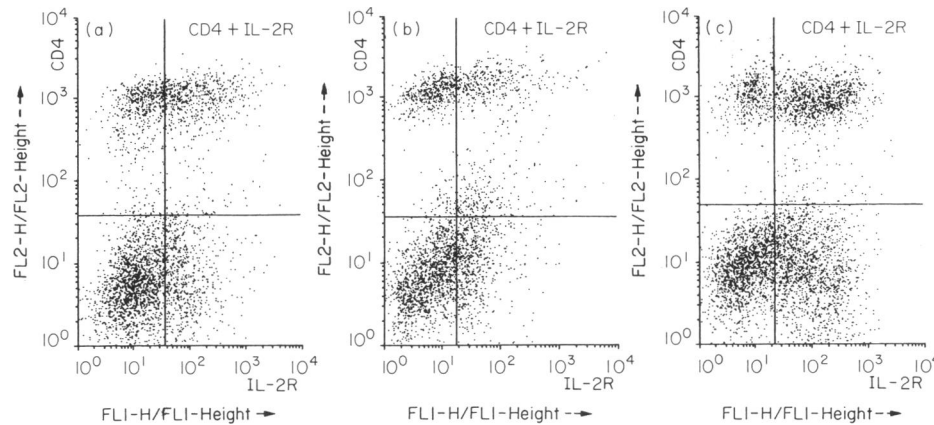


Fig. 3. FACS analysis of the expression of IL-2R on CD4⁺ cells from a naive animal after: (a) 48 h and (b) 7 days culture with infected cells (1 : 20). The percentage of CD4⁺ cells expressing IL-2R (50%) is unchanged between 48 h and 7 days. (c) IL-2R expression upon CD4 cells from the same animal is increased (to 70%) upon incubating infected cells: PBMC 1 : 10 for 48 h.

that many of these blasting cells express macrophage markers (J. Campbell, unpublished observations). At day 10, the blasting cells were 1C7⁺.

IL-A111 (anti-IL-2R) stained the cytoplasm of very few cells in sections from control nodes. However, within the day 4 draining node there were many IL-2R⁺ cells, grouped around, but not inside, the areas of blasting cells in the medulla (Fig. 4b). By day 8, the number of IL-2R⁺ cells had returned to control levels, disappearing completely by day 10.

Normal germinal centres (GC) had a mantle zone, characterized by small lymphocytes. MIB-1⁺ (Ki67) stained proliferating cells in an area at the 'base' of the GC analogous to the human GC dark zone [18]. VPM 30 staining was present only in well developed GC and generally did not stain the MIB-1⁺ areas in these GC, recognizing an area analogous to the human light zone [18]—both in its morphological appearance (Fig. 5a) and in being MIB-1⁺. Smaller GC with primarily 'dark zone' MIB-1⁺ cells did not stain with VPM30. By day 8, germinal centres could only be distinguished as having a mantle and dark zone. All VPM 30 staining was lost from GC, and only blasting MIB-1⁺ area cells persisted (Fig. 5b,c). No germinal centres were distinguishable by day 10.

DISCUSSION

Although *T. annulata* infections in naive cattle are usually fatal, parasite-specific CTL and serum antibodies have been detected in animals recovered from *T. annulata* infection [2,3]. Removing live parasite therefore removes any constraints upon the ability to generate immunity. In a previous report it was shown that infection of macrophages with *T. annulata* altered their antigen-presenting function *in vitro*, as infected cells induced proliferation of resting autologous T cells from naive animals [6,7]. Infected cells may therefore have an innate ability to activate T cells. In this study we have characterized activation of T cells by infected cells *in vitro* and examined the effects such activation may have upon immune response development *in vivo*. Here we show that infected cells can induce activation markers upon resting T cells *in vitro*, irrespective of their memory status. *In vivo*, T cells are not activated in con-

ventional sites. In addition, profound changes are seen in the development of germinal centres.

Expression of IL-2 and IL-2R genes is induced when T cells are activated [19,20]. Bovine T cells rapidly express IL-2R [21], and MHC class II [22] upon activation. In this study, both IL-2R and MHC class II were expressed on the surface of T cells when activated by the mitogen Con A. We show here that *T. annulata*-infected cells induce the expression of IL-2 receptors and MHC class II molecules on the surface of CD4⁺ and CD8⁺ T cells from animals which have not been infected with the parasite, and these naive PBMC are induced to proliferate by the infected cells. *Theileria annulata*-infected cells are therefore capable of producing enough stimuli to activate T cells in the absence of specific antigen.

When PBMC were separated from infected cells, T cell activation was no higher than that seen in PBMC incubated alone. Infected cells therefore require contact to activate T cells, and although soluble factors may be important they are not sufficient alone. The numbers of cells activated increased by adding more infected cells, and cells of both memory and 'naive' phenotypes were seen to be activated. It therefore seems likely that infected cells are capable of activating resting CD4⁺ or CD8⁺ T cells irrespective of phenotype, and the main limiting factor is infected cell: T cell contact.

The peak of proliferation induced by infected cells was similar to that induced by MHC class II associated antigen [10], whereas Con A-activated T cells showed maximum proliferation at day 2 (Fig. 2). This suggests that the 'non specific' activation of T cells by infected cells is not due to a parasite-associated mitogenic factor. Members of the 'superantigen' family of viral and bacterial peptides, and heat shock proteins which mimic mycobacterial antigens, have been associated with 'non-specific' activation of CD4⁺ and CD8⁺ T cells. The latter is also linked to an increase in expression of MHC class II on aberrant APC [23–25]. Antigens from *Trypanosoma cruzii* have been shown to induce strong proliferation of naive PBMC as the antigen can react with the majority of V β genes expressed by T cell receptors [26]. Given the kinetics of infected cell activation of T cells, an antigen presented in this manner is a more likely candidate than a mitogen or a superantigen.

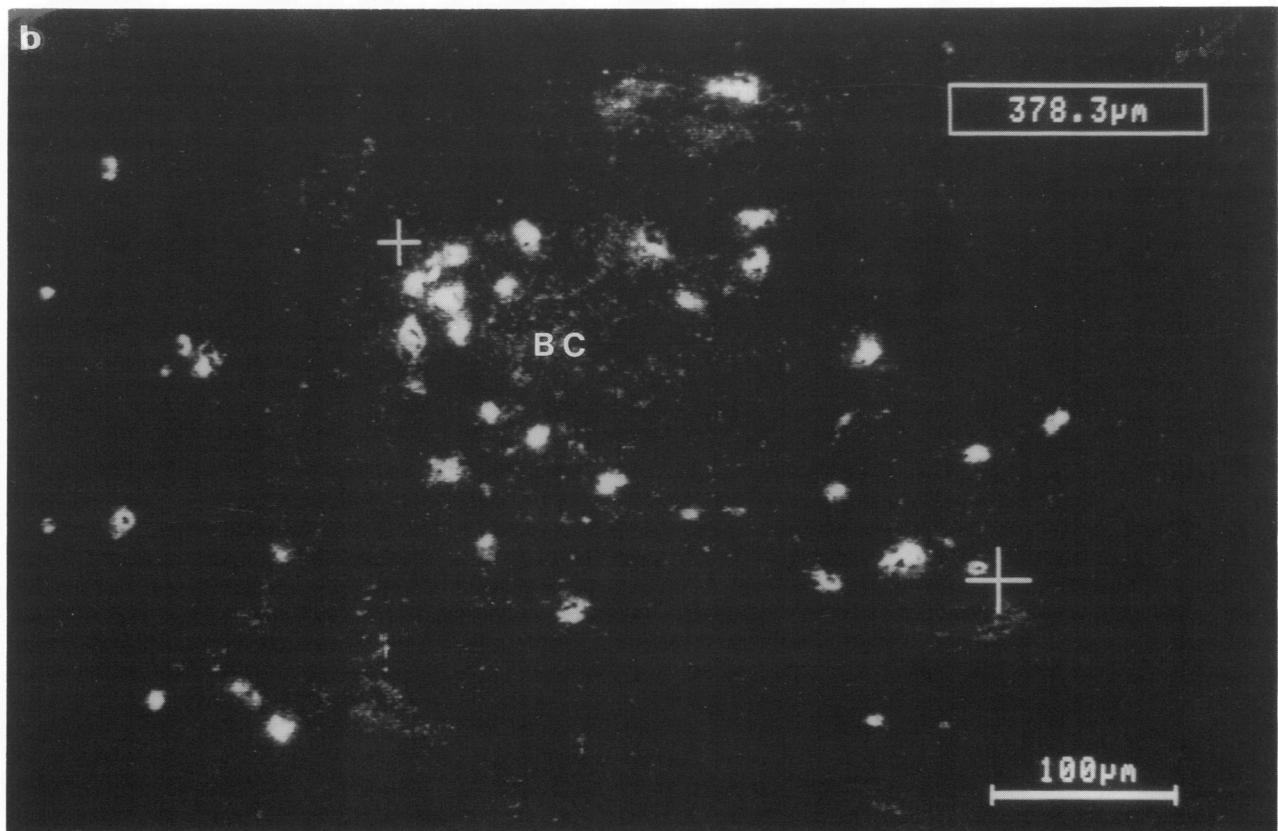
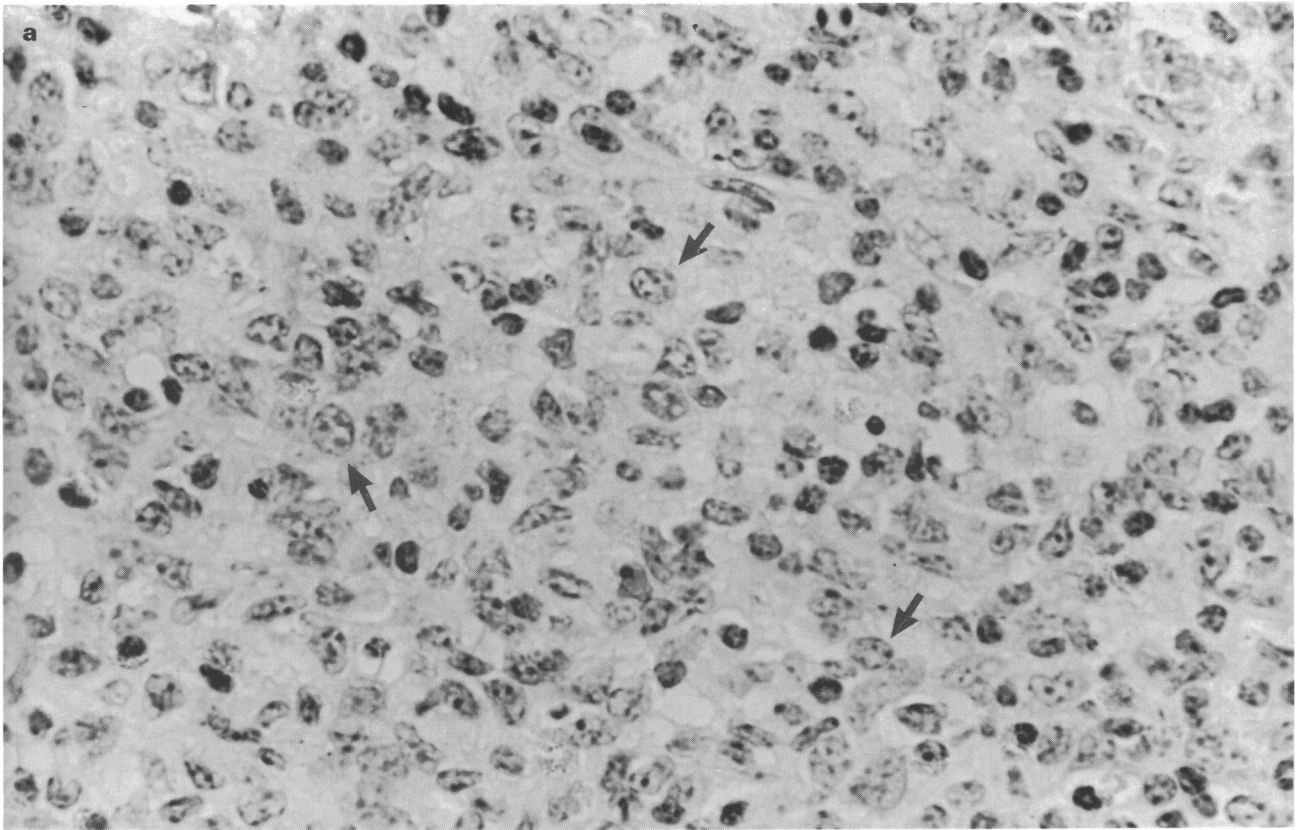


Fig. 4. Paraffin sections of bovine lymph node. (a) Large blasting cells which predominate throughout the draining lymph node 8 days post *Theileria annulata* infection. Examples are arrowed. (Haematoxylin and eosin, $\times 500$.) (b) Confocal micrograph using Vector red substrate and rhodamine filter. Distance between crosshairs is contained in box. IL-2R⁺ cells grouped around an area of blasting cells (BC; unstained by this method) in the medulla of a day 4 *T. annulata*-infected lymph node.

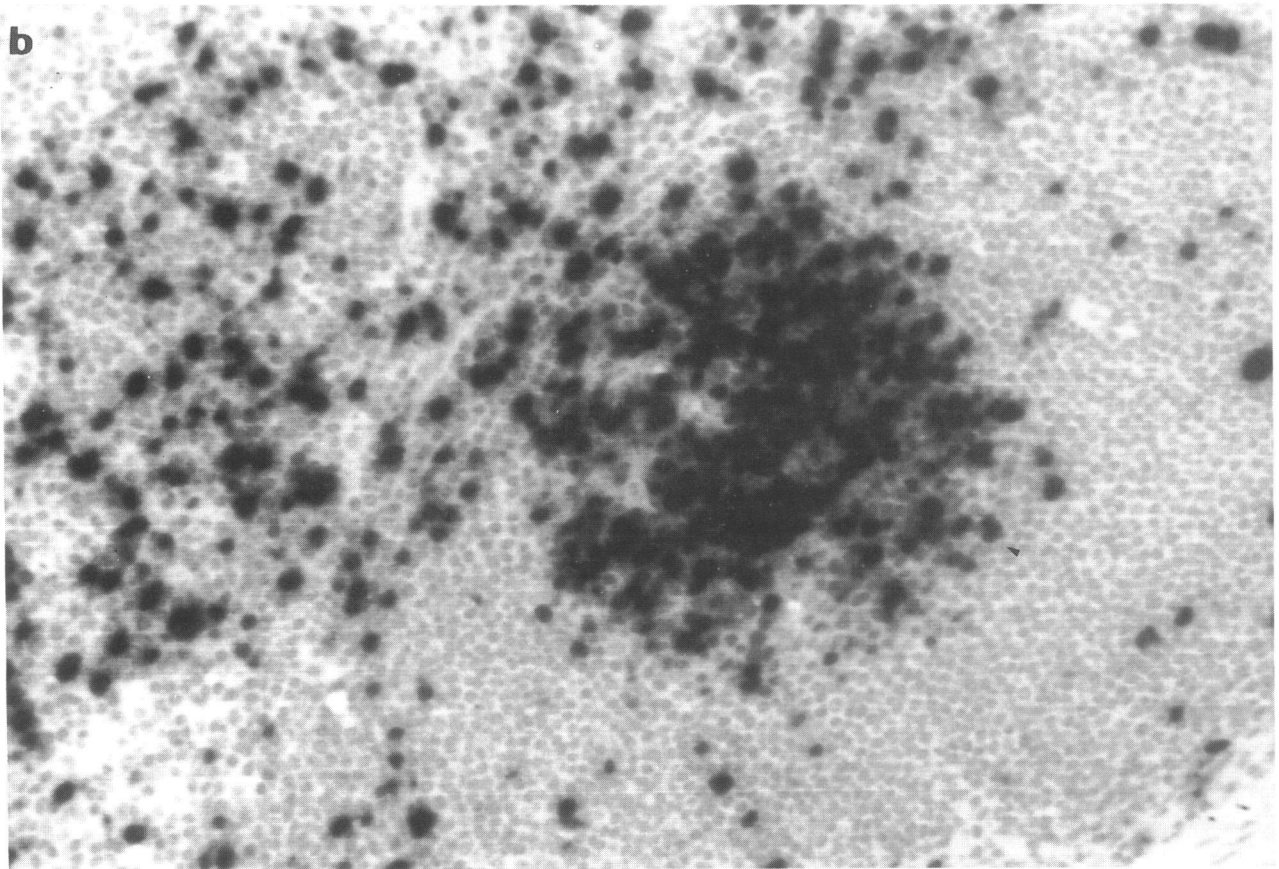
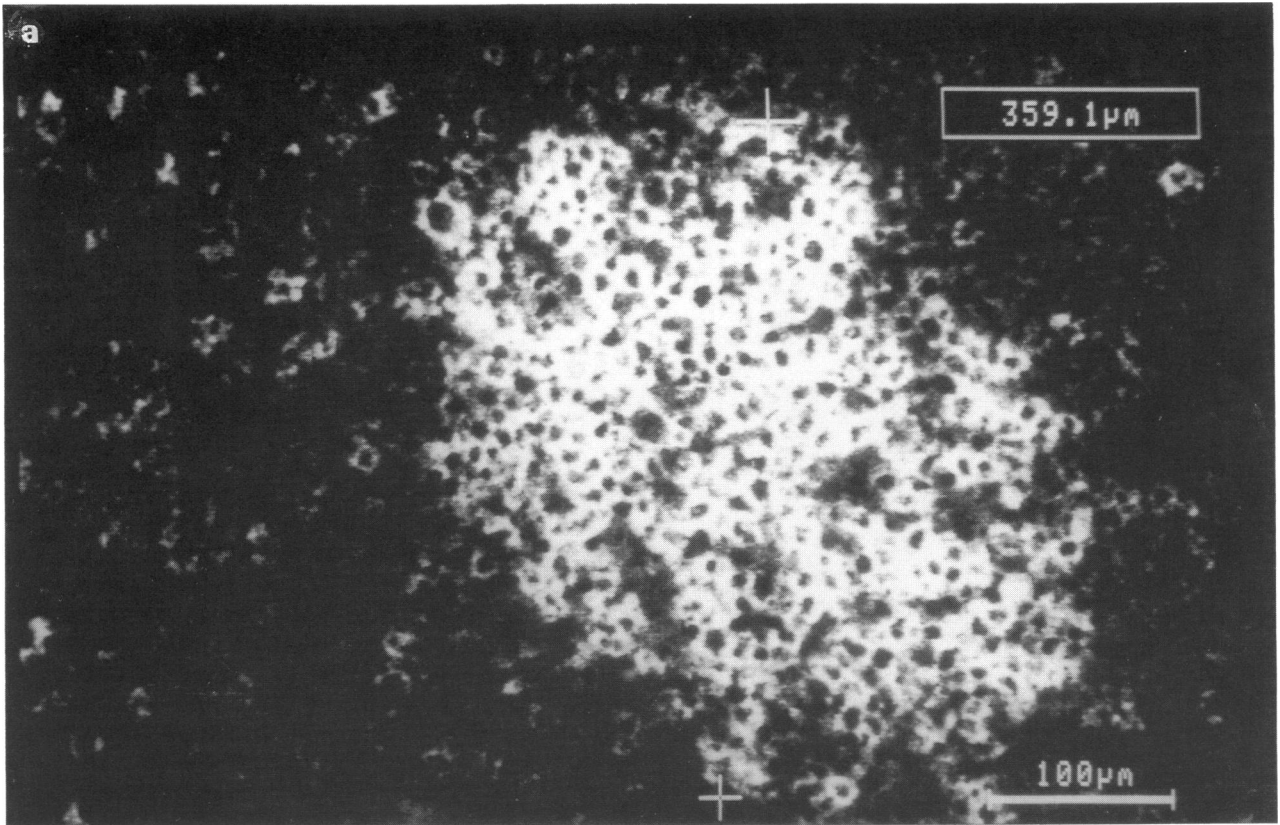


Fig. 5. Confocal micrographs using Vector red substrate and rhodamine filter. Distance between crosshairs is contained in box. (a) Normal bovine germinal centre (GC) stained with MoAb VPM30. The MoAb predominantly stains an area analogous to the human 'light zone', (b,c) Serial sections from a day 8 *Theileria annulata*-infected lymph node. Stained with (b) MIB-1 and (c) VPM30. Although MIB-1 'dark zone' staining persists, all VPM30 'light zone' staining is absent.

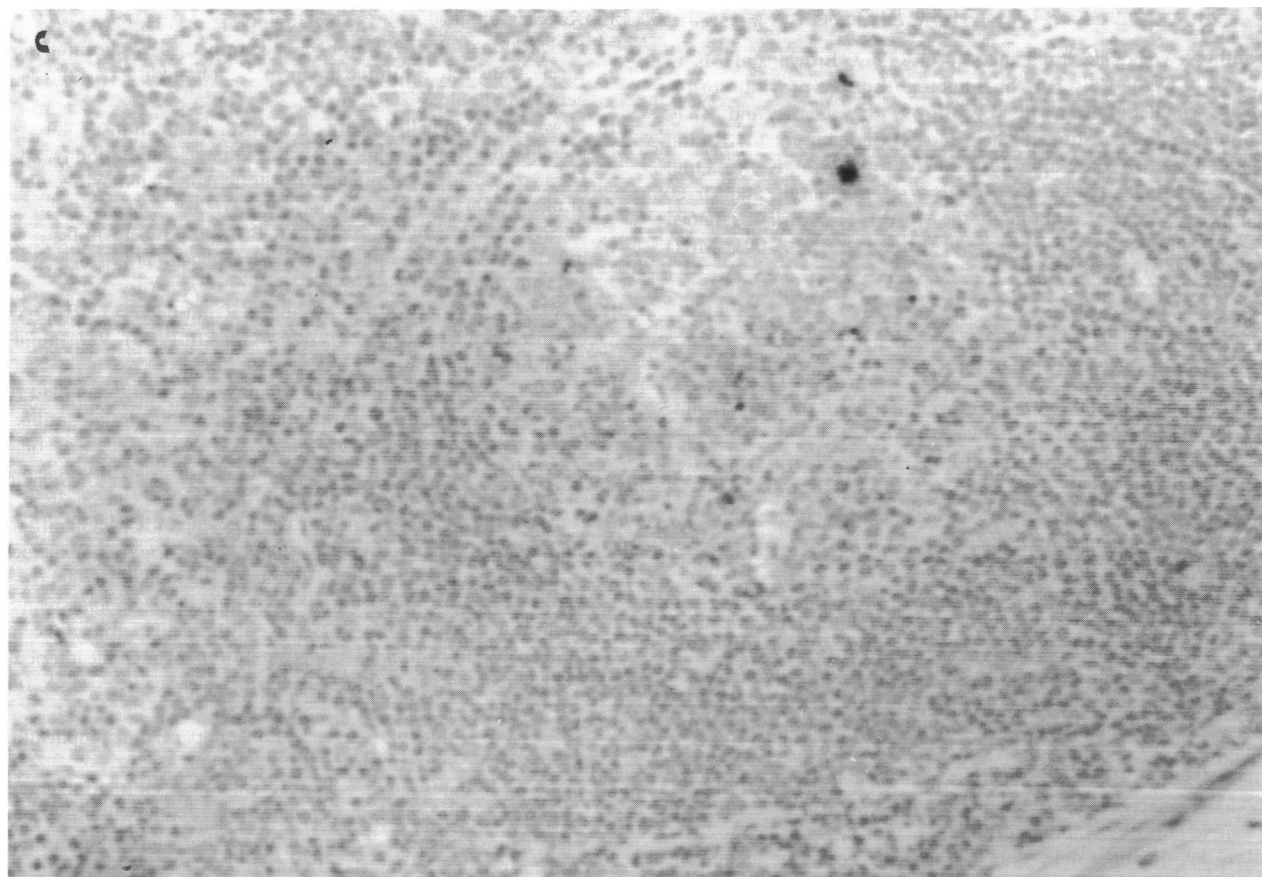


Fig. 5. Continued.

Further analysis of the activation of $CD4^+$ and $CD8^+$ subsets, and the antigens presented by MHC class II on infected cells, will be required in order to characterize further the antigenic element.

In the lymph nodes draining the sites of *T. annulata* infection, cells expressing IL-2R associated only with areas of parasite-induced proliferating cells in the medulla. Many of the latter cells are MHC class II⁺, express macrophage markers, and are found to be parasitized when schizonts are detectable. It seems likely that the *in vivo* appearance of IL-2R⁺ cells in the medulla reflects the 'non-specific' activation of T cells by parasitized cells *in vitro*. Antigen-primed interdigitating cells (IDC) have been shown to play a major part in primary bovine $CD4^+$ T cell responses [27]. In studies of the induction of primary immune responses to peptide antigens, it has been shown that T cells initially interact with these cells in the paracortex 4–7 days post-inoculation, in perivascular foci [28]. The appearance of IL-2R⁺ cells exclusively in the medulla suggests that *T. annulata* infection interferes with T cell activation in two ways: not only is T cell activation induced by the parasite-associated blasting cells, but also T cells are not being primed in normal anatomical sites. *Theileria annulata* infection therefore interferes with the normal pathways of T cell–APC interaction.

Germinal centres were severely affected by the parasite infection, initially losing the 'light zone', followed by total loss of morphology. This is indicative of aberrant T cell

function, as germinal centres are T cell-dependent [29]. The initial loss of the 'light zone' at a time when the proliferating dark zone is relatively unaffected also suggests that T cells are affected, as T cells are an essential component of the GC light zone [18,30], involved in cognate interactions to rescue somatically mutated (dark zone derived) centroblasts [31].

In this study we have shown that *T. annulata*-infected APC have the potential to interfere with immune response development as they can 'non-specifically' activate T cells irrespective of their antigen specificity *in vitro*. More importantly, a similar phenomenon is seen *in vivo* with the rapid appearance of activated T cells within the draining lymph node. Subsequent T cell priming in recognized sites of the lymph node does not occur. The alteration of T cell function is most dramatically manifested by the loss of the T cell-dependent compartments of germinal centres, followed by complete germinal centre breakdown.

Our findings also have important implications for vaccine design, as any vaccine would have to enhance the specific immune response without promoting non-specific proliferation. It seems unlikely that directing T helper cells towards direct interactions with infected cells would be effective, and indeed may be deleterious. We are currently studying the interactions of $CD4$ T cells from immune animals with infected cells and APC from within lymph nodes in order to determine which determinants are recognized by the 'specific' immune response.

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